THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 272, No. 22, Issue of May 30, pp. 14272–14276, 1997
Printed in U.S.A.

A Newly Identified Member of the Tumor Necrosis Factor Receptor Superfamily with a Wide Tissue Distribution and Involvement in Lymphocyte Activation*

(Received for publication, October 23, 1996, and in revised form, March 25, 1997)

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The tumor necrosis factor receptor (TNFR) superfamily consists of approximately 10 characterized members of human proteins. We have identified a new member of the TNFR superfamily, TR2, from a search of an expressed sequence tag data base. cDNA cloning and Northern blot hybridization demonstrated multiple mRNA species, of which a 1.7-kilobase form was most abundant. However, TR2 is encoded by a single gene which, maps to chromosome 1p36.22–36.3, in the same region as several other members of the TNFR superfamily. The most abundant TR2 open reading frame encodes a 283-amino acid single transmembrane protein with a 36-residue signal sequence, two perfect and two imperfect TNFR-like cysteine-rich domains, and a short cytoplasmic tail with some similarity to 4–1BB and CD40. TR2 mRNA is expressed in multiple human tissues and cell lines and shows a constitutive and relatively high expression in peripheral blood T cells, B cells, and monocytes. A TR2-Fc fusion protein inhibited a mixed lymphocyte reaction-mediated proliferation suggesting that the receptor and/or its ligand play a role in T cell stimulation.

The members of the tumor necrosis factor receptor (TNFR)[1]/nerve growth factor receptor (NGFR) superfamily are characterized by the presence of three to six repeats of a cysteine-rich motif that consists of approximately 30–40 amino acids in the extracellular part of the molecule (1). The crystal structure of TNFR-I complexed with its ligand showed that a cysteine-rich motif (TNFR domain) was composed of three elongated strands of residues held together by a twisted ladder of disulfide bonds (2). These receptors contain a hinge-like region immediately adjacent to the transmembrane domain, characterized by a lack of cysteine residues and a high proportion of serine, threonine, and proline, which are likely to be glycosylated with *O*-linked sugars. A cytoplasmic part of these molecules shows limited sequence similarities, a finding that may be the basis for diverse cellular signaling. At present, the members identified from human cells include CD40 (3, 4), 4–1BB (5), OX-40 (6), TNFR-I (7, 8), TNFR-II (9), CD27 (10), Fas (11), NGFR (12), CD30 (13), and LTBR (14). Viral open reading frames encoding soluble TNFRs have also been identified, such as SFV-T2 (9), Va53 (15), G4RG (16), and crmB (17).

Recent studies have shown that these molecules are involved in diverse biological activities such as immunoregulation (18, 19), by regulating cell proliferation (20–22), cell survival (23–25), and cell death (26–28).

Because of their biological significance and the diverse membership of this superfamily, we predicted that there would be further members of the superfamily. By searching an EST data base, we identified a new member of the TNFR superfamily. We report here the initial characterization of the molecule called TR2.

MATERIALS AND METHODS

Identification and Cloning of New Members of the TNFR Superfamily—An EST cDNA data base, obtained from over 500 different cDNA libraries (29, 30), was screened for sequence homology with cysteine-rich motif of the TNFR superfamily, using the blastn and tblastn algorithms (31). One EST was identified in a human T cell line library, which showed significant sequence identity to TNFR-II at the amino acid level. This sequence was used to clone the missing 5′ end by RACE (rapid amplification of cDNA ends) using a 5′-RACE ends-ready cDNA from human leukocytes (Clontech, Palo Alto, CA). This sequence matched three further ESTs (HTOBH42, HTOAU65, and HLHA49). Complete sequencing of these and other cDNAs indicated that they contained an identical open reading frame homologous to the TNFR superfamily, and it was named TR2. Analysis of several other ESTs and cDNAs indicated that some cDNAs had additional sequences inserted into the open reading frame identified above and might represent various partially spliced mRNAs.

Cells—The myeloid and B cell lines studied represent cell types at different stages of the differentiation pathway. KG1a and PLB 985 (32, 33) were obtained from Phillip Koeffler (UCLA School of Medicine), BJA-B was from Z Jonak (SmithKline Beecham), and TF 274, a stromal cell line exhibiting osteoblastic features, was generated from the bone marrow of a healthy male donor.[2] All of the other cell lines were obtained from the American Type Culture Collection (Rockville, MD). Monocytes were prepared by differential centrifugation of peripheral blood mononuclear cells (PBMC) and adhesion to tissue culture dish. $CD19^+$, $CD4^+$, and $CD8^+$ were isolated from PBMC by immunomag-

* This work was supported by National Institutes of Health Grants AI 28175 and AR 40248 (to B. S. K.) and by a postdoctoral fellowship from the American Heart Association, Indiana Affiliate (to S. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TNFR, tumor necrosis factor receptor; NGFR, nerve growth factor receptor; CHO, Chinese hamster ovary; DAPI, 4,6-diamidino-2-phenylindole; EST, expressed sequence tag; FISH, fluorescein *in situ* hybridization; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell; PHA, phytohemagglutinin; PMA, phorbol myristic acetate; RACE, rapid amplication of cDNA ends; mAb, monoclonal antibody; HVEM, herpesvirus entry mediator.

[2] K. B. Tan and Z. Jonak, unpublished data.

netic beads (Dynal, Lake Success, NY). Endothelial cells from human coronary artery were purchased from Clonetics (San Diego, CA).

RNA and DNA Blot Hybridization—Total RNA of adult tissues was purchased from CLONTECH or extracted from primary cells and cell lines with TriReagent (Molecular Research Center, Inc., Cincinnati, OH). 5–7.5 μg of total RNA was fractionated in a 1% agarose gel containing formaldehyde, as described (34), and transferred quantitatively to Zeta-Probe nylon membrane (Bio-Rad) by vacuum blotting. The blots were prehybridized, hybridized with ^{32}P-labeled *XhoI/Eco*RI fragment of TR2 or OX-40 probe, washed under high stringency conditions, and exposed to x-ray films.

High molecular weight human DNA was digested with various restriction enzymes and fractionated in 0.8% agarose gel. The DNA was denatured, neutralized, and transferred to nylon membrane and hybridized to ^{32}P-labeled TR2 or its variant cDNA.

In Situ Hybridization and FISH Detection—The *in situ* hybridization and FISH detection of TR2 location in human chromosomes were performed as described previously (35, 36). FISH signals and the DAPI banding pattern were recorded separately by taking photographs, and the assignment of the FISH mapping data with chromosomal bands was achieved by superimposing FISH signals with a DAPI-banded chromosome (37).

Production of Recombinant TR2-Fc Fusion Proteins—The 5′ portion of the TR2 containing the entire putative open reading frame of extracellular domain was amplified by polymerase chain reaction (38). For correctly oriented cloning, a *Hin*dIII site on the 5′ end of the forward primer and a *Bgl*II site on the 5′ end of the reverse primer were created. The Fc portion of human IgG_1 was PCR-amplified from ARH-77 (ATCC) cell RNA and cloned in the *Sma*I site of the pGem7 vector (Promega, Madison, WI). The Fc fragment, including hinge, CH_2, and CH_3 domain sequences, contained a *Bgl*II site at its 5′ end and an *Xho*I site at its 3′ end. The *Hin*dIII-*Bgl*II fragment of TR2 cDNA was inserted upstream of human IgG_1Fc and an in-frame fusion was confirmed by sequencing. The TR2-Fc fragment was released by digesting the plasmid with *Hin*dIII-*Xho*I and cloned into pcDNA3 expression plasmid.

The TR2-Fc plasmid, linearized with *Pvu*I, was transfected into NIH 3T3 by the calcium phosphate co-precipitation method. After selection in 400 μg/ml G418, neomycin-resistant colonies were picked and expanded. Enzyme-linked immunosorbent assay with anti-human IgG_1 and Northern analysis with ^{32}P-labeled TR2 probe were used to select for clones that produce high levels of TR2-Fc in the supernatant. In some experiments, a slightly differently engineered TR2-Fc produced in Chinese hamster ovary (CHO) cells was used. The TR2-Fc was purified by protein G chromatography, and the amino acid sequence of the N terminus was determined by automatic peptide sequencer (ABI).

In Vitro Transcription and Translation—The full-length TR2 cDNA was inserted into *Hin*dIII-*Xho*I sites of pcDNA 3 vector (Invitrogen, San Diego, CA). TNT-coupled reticulocyte lysate system (Promega) was used to *in vitro* transcribe and translate the TR2 cDNA in pcDNA 3. The ^{35}S-labeled reaction product was fractionated on a 5–15% gradient SDS-polyacrylamide gel, transferred onto an Immobilon membrane (Millipore, Bedford, MA), and exposed to x-ray film.

Blocking MLR-mediated PBMC Proliferation—PBMC were isolated from three healthy adult volunteers by Ficoll gradient centrifugation at 400 × *g* for 30 min. PBMCs were recovered, washed in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, 300 μg/ml L-glutamine, and 50 μg/ml gentamycin, and adjusted to 1×10^6 cells/ml for two donors and to 2×10^5 cells/ml for the third donor.

Fifty μl of each cell suspension was added to 96-well (round bottom) plates (Falcon, Franklin Lakes, NJ) together with 50 μl of TR2-Fc, IL-5R-Fc, anti-CD4 mAb, or control mAb. Plates were incubated at 37 °C in 5% CO_2 for 96 h. One μCi of [^{3}H]methylthymidine (ICN Biomedicals, Costa Mesa, CA) was then added for an additional 16 h. Cells were harvested, and radioactivity was counted.

RESULTS AND DISCUSSION

TR2 Is a New Member of the TNFR Superfamily—Fig. 1*a* shows the amino acid sequence of TR2 deduced from the longest open reading frame of one of the isolated cDNAs (HLHA49). Comparison with other sequenced cDNAs and with ESTs in the data base indicated potential allelic variants that resulted in amino acid changes at positions 17 (either Arg or Lys) and 41 (either Ser or Phe) of the protein sequence.

The open reading frame encodes 283 amino acids with a calculated molecular weight of 30,417. The TR2 protein was expected to be a receptor. Therefore, the potential signal se-

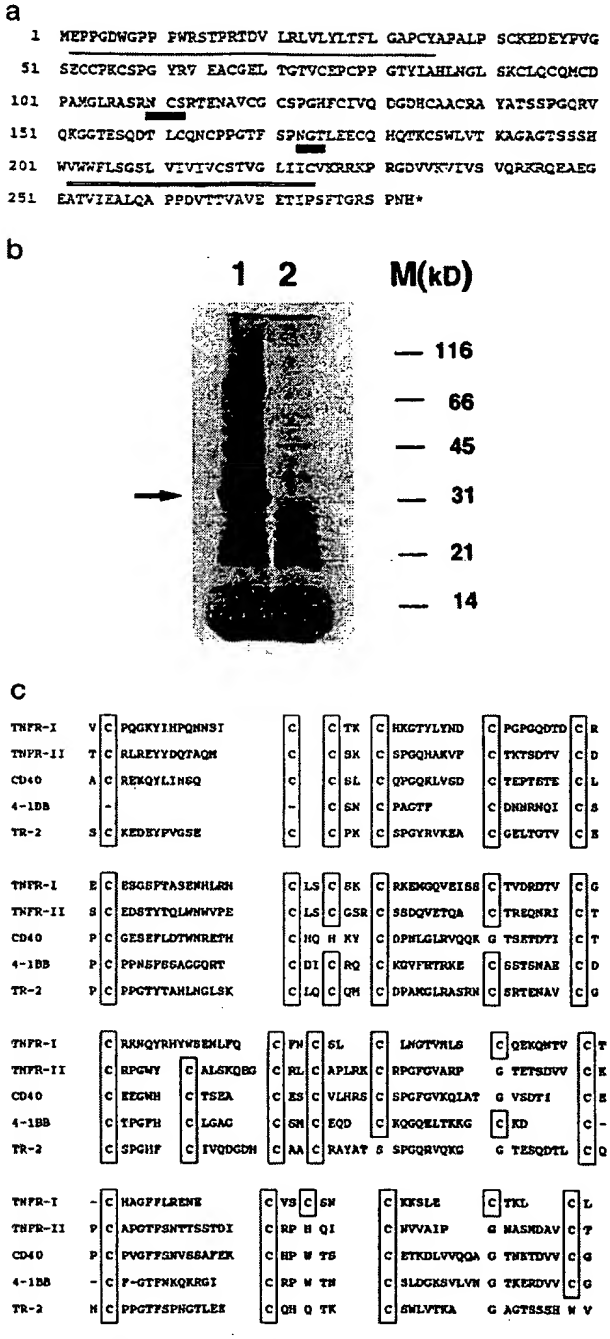

FIG. 1. *a*, deduced amino acid sequence of TR2. The signal region is *underlined*. The potential glycosylation sites are *underlined* with a *heavy line*. The putative transmembrane region is *double underlined*. The partial amino acid sequence of recombinant TR2 reads as *PALP*. . ., which indicates that the first 36 amino acids constitute a signal sequence. The GenBank™ accession number of this sequence is U81232. *b*, *in vitro* transcription and translation of TR2 cDNA. TR2 cDNA in pcDNA 3 (*lane 1*) and reversely oriented TR2 cDNA in pcDNA 3 (*lane 2*) was used for *in vitro* translation by the TNT-coupled reticulocyte lysate system (Promega). [^{35}S]Met-labeled translation product was fractionated by a 5–15% gradient SDS-polyacrylamide gel electrophoresis. *M* indicates molecular size markers, and the *arrow* indicates TR2 protein band. *c*, aligned amino acid sequence of extracellular motif of TR2 with other TNFR family members. The amino acid sequence of TR2 was aligned with those of TNFR-I, TNFR-II, CD-40, and 4–1BB on the basis of sequence homology and conserved cysteines.

quence and transmembrane domain were sought. A hydrophobic stretch of 23 amino acids toward the C terminus (amino acids 203–225) was assigned as a transmembrane domain, because it made a potentially single helical span (Fig. 1*a*), but the signal sequence was less obvious. The potential ectodomain of TR2 was expressed in NIH 3T3 and CHO cells as a Fc-fusion protein, and the N-terminal amino acid sequence of the recombinant TR2-Fc protein was determined in both cases. The N-terminal sequence of the processed mature TR2 started from amino acid 37, indicating that the first 36 amino acids constituted the signal sequence (Fig. 1*a*).

As shown in Fig. 1*b*, the *in vitro* translation product of TR2 cDNA was 32 kDa in molecular size. Since the first 36 amino acids constituted signal sequence, and its calculated molecular size was ~4 kDa, the molecular size of the protein backbone of processed TR2 would be approximately 28 kDa. Recently, Montgomery *et al.* (39) published a herpesvirus entry mediator (HVEM) whose cDNA sequence was identical to TR2. They found that the transfected HVEM cDNA produced a 32–36-kDa protein. Since it is larger than the *in vitro* product, this suggests that the protein is modified posttranslationally. Two potential asparagine-linked glycosylation sites are located at amino acid positions 110 and 173, as indicated in Fig. 1*a*.

Along with the other members of the TNFR family, TR2 contains the characteristic cysteine-rich motifs that have been shown by x-ray crystallography (2) to represent a repetitive structural unit. Fig. 1c shows the potential TNFR domain aligned among TR2, TNFR-I, TNFR-II, CD40, and 4–1BB. TR2 contained two perfect TNFR motifs and two imperfect ones.

The TR2 cytoplasmic tail (TR-2 cy) does not contain the death domain seen in the Fas and TNFR-I intracellular domains, and appears to be more related to those of CD40cy and 4–1bbcy. Signals through 4–1BB and CD40 have been shown to be co-stimulatory to T cells and B cells, respectively (40, 41).

TR2 RNA Expression—A human tissue RNA blot was used to determine tissue distribution of TR2 mRNA expression. TR2 mRNA was detected in several tissues with a relatively high level in the lung, spleen, and thymus, but was not found in the brain, liver, or skeletal muscle (Fig. 2*a*). TR2 was also expressed in monocytes, $CD19^+$ B cells, and resting or PMA plus PHA-treated $CD4^+$ or $CD8^+$ T cells. It was only weakly expressed in bone marrow and endothelial cells (Fig. 2*b*), although expression was observed in the hematopoietic cell line KG1a. For comparison, the tissue distribution of OX-40, another member of the TNFR superfamily, was examined. Unlike TR2, OX-40 was not detected in the tissues examined and was detected only in activated T cells and KG1a. Several cell lines were negative for TR2 expression, including TF274 (bone marrow stromal), MG63, TE85 (osteosarcomas), RL 95–2 (endometrial sarcoma), MCF-7, T-47D (breast cancer cells), BE, HT 29 (colon cancer cells), HTB-11, and IMR-32 (neuroblastomas), although TR2 was found in the rhabdosarcoma HTB-82 (data not shown).

Several cell lines were examined for inducible TR2 expression. HL60, U937, and THP1, which belong to the myelomonocytic lineage, all increased TR2 expression in response to the differentiating agents PMA or Me_2SO (Fig. 2*c*). Increases in expression in response to these agents were also observed in KG1a and Jurkat cells. In contrast, PMA did not induce TR2 expression in MG63, but unexpectedly TNF-α did.

In almost all cases, the predominant mRNA was approximately 1.7 kilobases in size, although several higher molecular weight species could be detected in some tissues (Fig. 2*a*), and many cDNAs and ESTs that were sequenced contained insertions in the coding region indicative of partial splicing. The abundance of higher molecular weight mRNAs raises the possibility that TR2 may in part be regulated at the level of mRNA maturation.

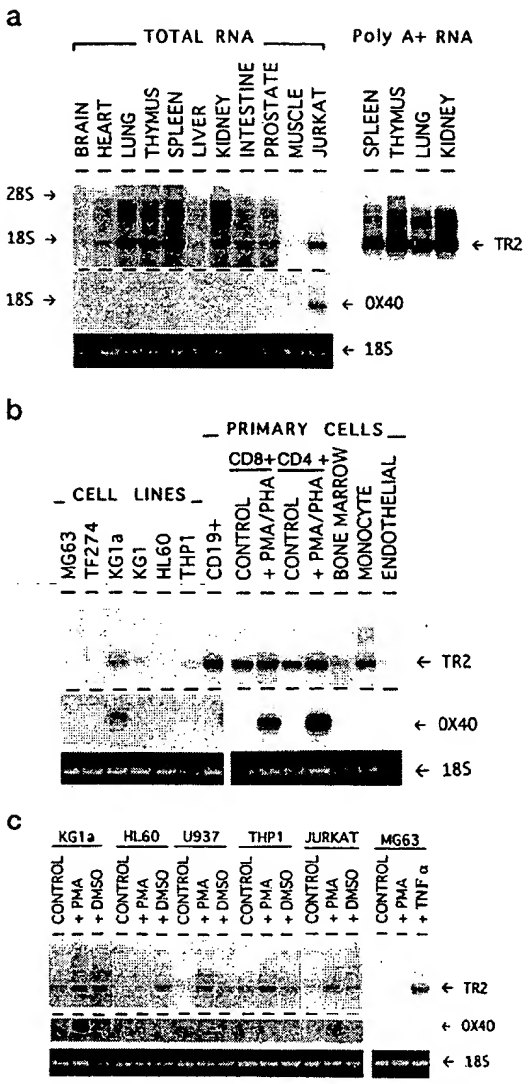

FIG. 2. *a*, Northern blot tissue distribution of TR2 and OX-40. RNA extracted from Jurkat cells, treated with 50 nM PMA for 48 h, was electrophoresed together with the tissue samples for comparison. The poly(A^+) RNA was purified from 10 μg of total RNA using Dynabeads oligo(dT) 25. A photograph of the ethidium bromide-stained 18 S ribosomal RNA is included to show the RNA loading of the various samples. Under the electrophoretic conditions used, TR2 (*arrow*), OX-40, and 18 S ribosomal RNAs have apparent sizes of 1.7, 1.3, and 1.8 kilobases, respectively. *b*, Northern blot RNA expression in cell lines and primary cells. Total RNA was extracted from untreated or cells treated for 48 h with PMA (10 ng/ml) and PHA (5 μg/ml) and 5 μg of each sample was analyzed. *c*, Northern blot-induction of RNA expression. KG1a, HL60, U937, THP1, and Jurkat cells were treated with 50 nM PMA or 1.5% Me_2SO for 70 h, and total RNA was extracted for analysis. MG63 cells were treated with 50 nM PMA or 100 ng/ml of TNFα for 7 h.

TR2 Maps at 1P36.2-P36.3—The FISH mapping procedure was applied to localize the TR2 gene to a specific human chromosomal region. The assignment of a hybridization signal to the short arm of chromosome 1 was obtained with the aid of DAP I banding. A total of 10 mitotic figures were photographed, one of which is shown in Fig. 3*a*. The double fluorescent signals are indicated on the schematic diagram of chromosome 1 as shown in Fig. 3*b*. This result indicated that the TR2 gene is located on the chromosome 1 region p36.2-p36.3. The TR2

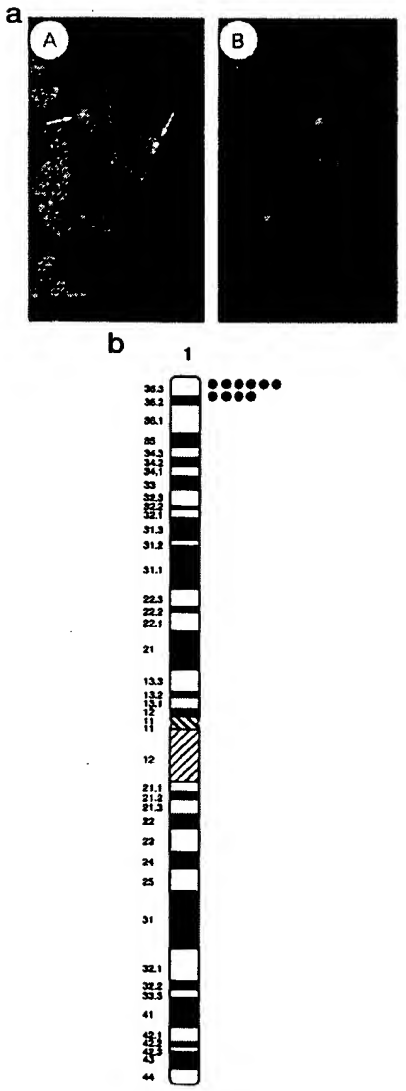

FIG. 3. *a*, combined DAPI banding and *in situ* hybridization. *Panel A*, the position of the TR2 probe is shown by *orange dots* on a chromosome. *Panel B*, the same chromosome with DAPI banding. *b*, localization of TR2 on human chromosome. A schematic diagram of human chromosome 1 is shown, and the positions at which TR2 hybridization signals were detected are indicated by *filled circles*.

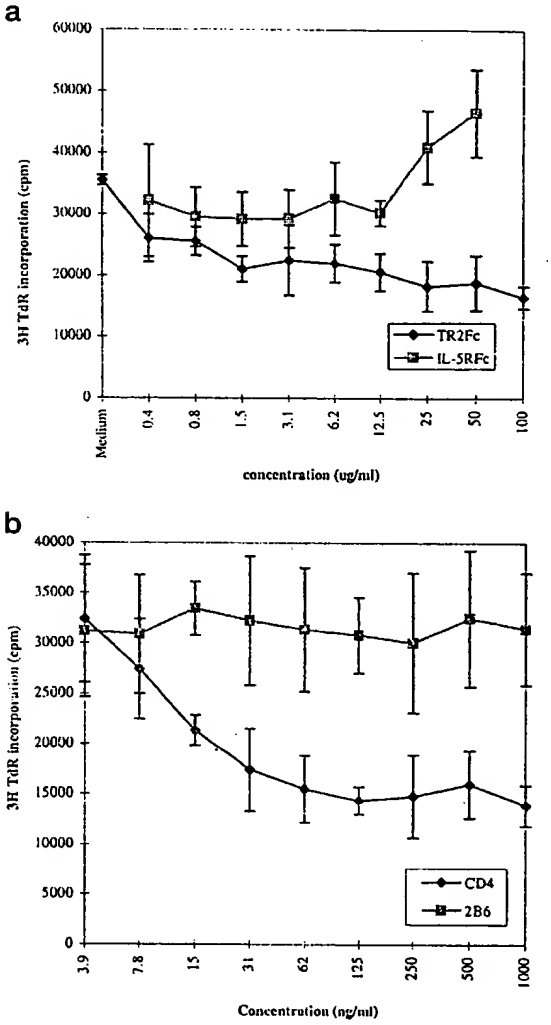

FIG. 4. **Inhibition of allogenic proliferation by TR2-Fc.** *a*, effect of TR2-Fc on a three-way MLR. PBMCs were adjusted to 1×10^6 cells/ml for two donors and to 2×10^5 cells/ml for the third donor. Fifty microliters of each cell suspension was added to 96-well round bottom plates, together with 50 μl of TR2-Fc (○) or with 50 μl of IL-5R-Fc (■). [^{3}H]Methylthymidine incorporation was measured as an indication of cell proliferation. The *error bars* represent standard errors. *b*, effect of anti-CD4 mAb on a three-way MLR. PBMCs were adjusted to 1×10^6 cells/ml for two donors and to 2×10^5 cells/ml for the third donor. Fifty microliters of each cell suspension was added to 96-well round bottom plates together with 50 μl of anti-CD4 mAb (○) or with 50 μl of 2B6 mAb (■). [^{3}H]Methylthymidine incorporation was measured as an indication of cell proliferation. The *error bars* represent standard errors.

position is in close proximity with CD30 (42), 4–1BB (43, 44), OX-40 (45), and TNFR-II (46), suggesting that it evolved through a localized gene duplication event. Interestingly, all of these receptors have stimulatory phenotypes in T cells in response to cognate ligand binding, in contrast to Fas and TNFR-I, which stimulate apoptosis. This prompted us to test if TR2 might be involved in lymphocyte stimulation.

TR2-Fc Interferes with MLR-mediated Proliferation of PBMC—To determine the potential involvement of cell surface TR2 with its ligand in lymphocyte proliferation, we examined allogeneic MLR proliferative responses. As shown in Fig. 4, *a* and *b*, when TR2-Fc was added to the culture, a significant reduction of maximal responses was observed ($p < 0.05$). The addition of TR2-Fc at 100 μg/ml inhibited the proliferation up to 53%. No significant inhibition of proliferation was observed with the control IL-5R-Fc. Surprisingly, at high concentrations (10–100 μg/ml) IL-5R-Fc was shown to enhance proliferation. The concentrations of TR2-Fc required to inhibit MLR proliferation (1–100 μg/ml) are comparable with those of CD40-Fc required for inhibition in other lymphocyte assays (47–50). An anti-CD4 mAb assayed simultaneously inhibited MLR-mediated proliferation up to 60%, whereas a control anti-IL-5 mAb failed to inhibit the proliferation. It is well known that a major component of the MLR proliferative response is T cell-dependent; hence, it would appear that inhibiting the interaction of TR2 with its ligand prevents optimal T lymphocyte activation and proliferation.

Hence, we have identified an additional member of the TNFR superfamily that either plays a direct role in T cell stimulation or binds to a ligand which can stimulate T cell proliferation through one or more receptors, which may include TR2. We are currently trying to identify this ligand to which TR2 binds to clarify its role.

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